J Mater Sci: Mater Med (2009) 20:397–404
DOI 10.1007/s10856-008-3586-z

Enhancing growth and proliferation of human gingival fibroblasts on chitosan grafted poly (ε-caprolactone) films is influenced by nano-roughness chitosan surfaces

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Received: 18 January 2007 / Accepted: 4 September 2008 / Published online: 25 September 2008

Abstract The bioactivity of poly (ε-caprolactone) (PCL) films is improved by grafting chitosan (CS) surfaces with various values of nano-roughness on PCL surfaces. To examine the effects of the design, growing human gingival fibroblasts (HGFs) on the films was conducted. Various values of nano-rough CS surfaces were cast using nano-rough PCL molds that had been fabricated using a solvent-etched technique. The features of nano-CS/PCL surfaces were characterized using an atomic force microscope (AFM) to observe the topography and to determine the value of centerline average roughness of a surface, R_a. The R_a values of the nano-CS/PCL films were 36.8 ± 1.6, 100.0 ± 3.0, and 148 ± 7.0 nm, while that of the smooth CS/PCL film was 12.5 ± 1.6 nm. The growth and proliferation of HGFs on the films are elucidated by fluorescent staining and analyzed by MTT viability assay following three and 7 days of culture. The viability assay of the cells reveals that the growth rates of HGFs on both CS/PCL and nano-CS/PCL films significantly exceed (95% or more; $P < 0.001$) those of PCL on both days, demonstrating the improvement of the bioactivity of PCL films by grafting CS. Additionally, the growth rates and proliferations of HGFs on nano-CS/PCL films of roughness 100 and 148 nm markedly exceed (15% or more; $P < 0.001$) those on 36.8 nm nano-CS/PCL and CS/PCL films, after both periods of culturing, indicating that the high nano-roughness CS surfaces further enhance the growth rate of HGFs. In conclusion, markedly improving the bioactivity of PCL films by grafting CS is demonstrated. Moreover, high nano-roughness of nano-CS/PCL films can further accelerate the growth and proliferation of HGFs compared with those of CS/PCL films. This work presents a new concept for designing biomaterials in tissue engineering.

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1 Introduction

Using synthetic polymer scaffolds to repair various damaged tissues has been investigated by numerous researchers [1, 2]. One of the design concepts applied in preparing a useful bioactive polymer scaffold is that the base polymers are compatible with cells whereas the cell-adhesive characteristics of scaffolds are dominated by immobilized bioactive factors, such as RGD peptides [3, 4]. In this respect, grafting or blending a bioactive factor onto the surfaces of polymer films or scaffolds has been extensively studied [4–6]. The modification of the physical characteristics of the surfaces of biopolymers, including by enhancing the hydrophilic properties of the surfaces; changing the sizes of the pores in the materials, and increasing the roughness of the surfaces [2, 7–9], have also been suggested and have been demonstrated to enhance the growth of cells on the biomaterials.

Although PCL polymer has been applied in such clinical applications as sutures [2], it does not easily interact with bio-specialized cells of soft tissues because it has a highly

crystalline structure. However, its structure has great potential for use in the engineering of hard tissues, such as in bone regeneration, by the addition of various bioactive factors, such as hydroxyapatite particles or collagen mats, into its matrix [10, 11]. The authors' earlier work described the modification of PCL surfaces with a poly (ethylene glycol)-grafted RGD peptide to promote the growth rate of endothelial cells [12]. However, the modifications of the inert properties of PCL with chitosan which is further processed with nano-roughness treatments for engineering soft tissues are few.

Chitosan (CS), an amino polysaccharide (poly-1, 4D-glucosamine), is derived from chitin by de-acetylation. Since CS is both non-toxic and biocompatible, it has been extensively used in medical applications, including wound dressings and drug delivery systems [13–15]. Additionally, CS has both reactive amino and hydroxyl groups that serve as bioactive factors, which can be readily chemically modified to promote cell growth and proliferation [4, 16].

Recently, the effects of the nano- or micro-structure surface features on the growth of various cells have been examined [8, 9, 11, 17–21]. Some researchers have found that in the micro-structured surface environment, increasing the surface roughness by sand-blasting can affect the production of growth factors and the number of osteoblast-like MG-63 cells on a titanium surface [19, 20], or promote cell adhesion and migration on a PMMA surface [21]. However, the micro-structured surfaces of biodegradable polyesters do not facilitate the growth of vascular cells [9, 17]. Increasing the surface roughness of PU to 2–40 nm increases the growth rate of endothelial cells on the surface [8]. Other researchers have also reported that nano-structured surfaces can promote the growth of bladder smooth muscle cells or fibroblasts, although nano-roughness values of the surfaces of different groups are differences [9, 17, 18].

In this work, CS surfaces with various values of nano-roughness are fabricated and grafted as bioactive factors onto PCL films to yield nano-CS/PCL films on which the growth rates of fibroblasts might be enhanced by both CS and the selection of particular values of nano-roughness. The in vitro growths of HGFs from primary cells on CS/PCL films with or without various nano-feature treatments were further evaluated to examine the design. With respect to other characteristics of the nano-CS/PCL films, the nano-roughness of the films was determined using an atomic force microscope (AFM) to observe topologic micrographic images, and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was used to analyze the amide groups on the CS surfaces. The results of this work suggest that incorporating both a bioactive factor and a suitable nano-roughness surface feature to modify inert biopolymer films or scaffolds is a new approach for engineering soft tissues.

2 Materials and methods

2.1 Materials

PCL with an Mw. of 80 KDa was obtained from Aldrich Corp., USA. Chitosan with 96% de-acetylated was purchased from Primex Ingredients ASA, Norway (batch #: TM1540, Mw. of 200 KDa); it had a characteristic viscosity of 57 mPa.S when 1% CS was dissolved in 1% acetic acid solution. Dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from J. T. Baker, USA. Hexamethylene diisocyanate (HMDI) was obtained from ACROS, USA. Genipin powder was obtained from Challenge Bio-products Co., Taiwan, ROC.

2.2 Preparation of PCL mold with various nano-rough surfaces (Diagram 1)

Twenty weight percentage of PCL dissolved in THF was poured into a dish, and the solvent was then fully evaporated to yield a PCL film with a diameter of 4 cm and a thickness of 0.2 mm. Solvent etched-evaporation was employed with some modifications, to prepare a nano-rough PCL (nano-PCL) surface [12]. The film was etched using acetone at a spinning rate of 80 rpm, and the solvent was evaporated at 40°C. When the solvent was fully evaporated, the PCL film with a nano-rough surface was fabricated. A series of PCL molds with various surface roughness values were prepared by varying the temperature to control the rates of evaporation of the solvent. The various nano-PCL molds were fabricated to cast CS to yield nano-rough CS surfaces (Diagram 1).

2.3 Preparation of chitosan films with various nano-rough surfaces

In casting nano-roughness CS surfaces with various values of roughness using the aforementioned PCL molds, 2% CS was dissolved in an acetic acid solution that contained 1% genipin, a crosslinking agent, to cross-link the CS. The CS solution was crosslinked for 4–6 h at room temperature before it was cast. Genipin is a natural crosslinking agent, which has often been applied to cross-link the amine groups of biopolymers [22, 23]. When the solvent was fully evaporated, a series of genipin-crosslinked CS films with various values of nano-roughness were prepared.

2.4 Fabrication of CS/PCL films with CS surfaces with various values of nano-roughness

The side of the CS films opposite its nano-rough surface initially reacted with 10% HMDI in THF in the presence of stannous 2-ethyl-hexanoate at 50°C. After the films had

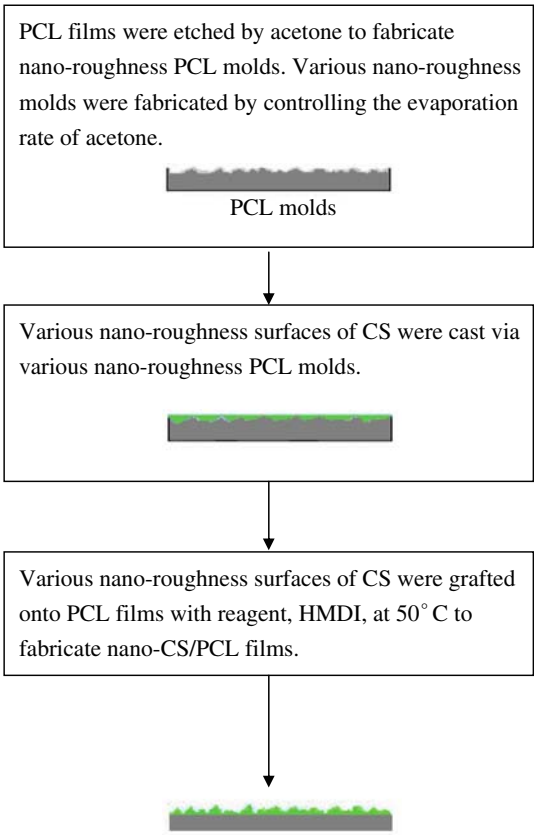

Diagram I The procedure for fabricating nano-CS/PCL films with various R_a values. Various degrees of etched nano-rough PCL molds were used to cast nano-CS surfaces

been rinsed three times with THF and dried with nitrogen, the HMDI-treated CS surfaces reacted with 10% of PCL/THF solution at 50°C to yield a nano-CS/PCL film. After the solvent had been evaporated, the nano-CS/PCL films were dried and then used for this study.

2.5 Observation of topography of nano-CS/PCL films by AFM

The topography and roughness of the surfaces of PCL, CS/PCL and various nano-roughness values of nano-CS/PCL films under dry conditions were observed using an AFM (Hitachi DI-5000, Hitachi Koki Co. Ltd, Japan). Five different surface areas (e.g., 100 μm^2/area) were scanned for each sample and images of the surfaces were obtained in tapping mode [8, 12]. The topography and the centerline average roughness of a surface, R_a, determined using the built-in software (Nanoscope IIIa, Digital Instruments, CA, USA) were obtained to specify the roughness of the surface [8, 17, 18, 21]. To examine the effects of hydration of the films on the nano-roughness of nano-CS/PCL surfaces, the films were hydrated in PBS for 24 h at 37°C and then slowly dried. The R_a values of the dry nano-rough surfaces of the aforementioned hydrated films were determined using the same AFM.

2.6 Characterization of CS surfaces by ATR-FTIR

The transmission spectra of the CS surfaces were obtained using an ATR-FTIR analyzer at a resolution of 2 cm^{-1}, and analyzed using a built-in standard software package (Perkin-Elmer Spectrum One, Perkin-Elmer Co., Norwalk, CT, USA).

2.7 HGFs cultured on PCL, nano-PCL, CS/PCL and nano-CS/PCL films

Human gingival fibroblasts (HGFs) were isolated and cultured as described elsewhere [24, 25]. In brief, the excised tissues and tooth were placed in 'explant' medium (DMEM with 10% FCS, gentamicin sulphate, 2.5 μg/ml fungizone, 100 units/ml penicillin, 100 μg/ml streptomycin, and non-essential amino acids). The excised gingiva was then cut into small pieces, and placed in tissue culture dishes to allow the establishment of explant cultures. Subsequent subcultures were made in DMEM that contained 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and non-essential amino acids. The culture the HGF cells were plated at an initial density of 2.0×10^4 viable cells/ml culture medium that contained 10% FCS into a 24-well tissue culture-treated plate. The cells were allowed to attach and spread for 24 h. At the end of this period and daily thereafter, the medium was removed from each well. Cells were used between the fourth and the eighth transfers. The details of the procedures have presented elsewhere [4, 8, 12].

One milliliter HGFs suspensions harvested from four to eight cycles of subcultures, with a cell density of 2.0×10^4 viable cells/ml were obtained and seeded onto a 24-well polystyrene tissue culture plate (Falcon, USA). The wells of the plate were covered with PCL, nano-PCL, CS/PCL and nano-CS/PCL films with various values of roughness to investigate the adhesion and growth of the cells on the films. Briefly, the tested films were cut to a size of 1 cm^2 and sterilized under UV light for one day and then washed in 70% alcohol. After the films had been rinsed in sterilized cultural medium, they were placed on the bottom of the tissue culture plate and covered with a sterilized Teflon ring to prevent floating. The culture wells were incubated at 37°C with 5% CO_2/95% of air and 90% relative humidity for 3 and 7 days. Additionally, the numbers of adhesive cells following 8 h of incubation are determined by subtracting the numbers of floating cells in the culture

medium from the original numbers of the seeded cells to observe the effect of materials on initial cell adhesion.

After the culture, the cells on the films were stained with propidium iodine (PI) (Sigma Chemicals, St. Louis, MO, USA), according to the procedures described in the authors' earlier works [4, 8, 12]. Their morphology was observed using a phase contrast microscope that was equipped with a fluorescent light source (Nikon TE-100, Japan). Photographs were taken using a CCD camera.

The viability of the cells was also measured using thiazolyl blue assay (MTT reagent, Sigma Chemicals, St. Louis, MO, USA) [26, 27] with minor modifications [4, 8, 12]. First, a 300 μl MTT solution was incubated with the cells in the polystyrene culture plate wells, and the wells were covered with PCL, CS/PCL, and nano-CS/PCL films with various values of roughness at 37°C for 4 h. Then, dimethyl sulfoxide solution (DMSO, Sigma Chemicals, St. Louis, MO, USA) was added to dissolve formazan crystals. The absorbance values of the formazan solutions, obtained from the aforementioned films, were measured using an ELISA micro-plate reader at 570 nm (ELx800, Bio-Tek Instruments, Inc., Winooski, Vermont, USA) [26]. The effect of PCL on the absorbance values was included in the background value: the absorbance of the formazan solution in the polystyrene cell culture well was also measured. For comparison, the ratios of the absorbance values of the formazan solutions in polystyrene cultural wells to those of PCL, nano-PCL, CS/PCL, and nano-CS/PCL films with various values of roughness are defined as the relative growth rates of the cells. All of the calculations were made using Sigmastat statistical software (Jandel Science Corp., San Rafael, CA, USA). A confidence level of at least 95% corresponded to statistical significance. Data are presented as mean ± SD., having been measured at least in triplicate.

3 Results and discussion

Biopolymer films are modified to promote cell growth for tissue engineering can be generally categorized using two types of approach. First, bioactive components such as hydroxyapatite particles are incorporated into polyesters by co-polymerization or blending [5, 10, 11]. Second, the biopolymers with nano-rough surfaces can be fabricated using various techniques [8, 17, 18]. This work combines the two types of approach by grafting CS with surfaces with various values of nano-roughness onto a PCL film. Moreover, the effects of the approach on the growth rates of HGFs are examined.

To etch PCL surfaces as molds with various nano-roughness values, various solvent compositions, etching conditions, and evaporating conditions were used [12]. Acetone was then chosen as a solvent with mild spinning, and the solvent evaporation temperature was set to 40°C to yield PCL molds. To fabricate a series of PCL molds with various values of nano-roughness, the temperature was varied to control solvent evaporation rate. Then, CS surfaces with various nano-roughness values were cast using the PCL molds (Diagram 1). After the nano-CS films reacted with HMDI/THF reagent, PCL solutions were added and reacted with the nano-CS films to fabricate nano-CS/PCL films with various values of nano-roughness.

The AFM topographies and surface features of CS/PCL films with various values of nano-roughness were similar to those in our earlier works [8, 28]. The R_a value has been adopted extensively to characterize the nano-roughness of surfaces [8, 18–21], and so is used herein. The R_a value of smooth CS/PCL film is 12.5 ± 1.7 nm and those of various nano-CS/PCL films are 148.0 ± 7.0 nm, 100.0 ± 3.0 nm, and 36.8 ± 1.6 nm which significantly exceed that of the smooth one. Moreover, the topographies of those surfaces are similar to authors' earlier works [28]. Since nano-rough surfaces were fabricated using other techniques with an R_a value of 40 nm, the surfaces positively affect the growth of HUVEC [8]. Accordingly, whether other cells such as HGFs are sensitive to the surfaces with similar values of nano-roughness or not is of interest.

CS contains amine groups which can be easily crosslinked by chemical agents to maintain the structure. Here, genipin was utilized as a crosslinking agent, which was extracted from a natural plant, to maintain the surface features of CS and maintain the bioactive properties of CS potentially to promote the growth of HGFs. The crosslinking of amine groups of biomaterials using genipin has been described and the crosslinked products have been demonstrated to be less toxic than glutaldehyde [22, 23]. According to the authors' earlier work [28], the crosslinked CS surfaces reach an equilibrium state of hydration within 12 h. In this work, the nano-CS/PCL films were hydrated for 24 h, then slowly dried and examined the nano-roughness by AFM (Fig. 1a–d) to indirectly investigate the effects of hydration on the nano-rough surfaces. The R_a values of the aforementioned the smooth CS/PCL surface and three hydrated CS/PCL surfaces are 19.1 ± 1.5 nm (Fig. 1a) and 78.5 ± 6.3 nm, 36.9 ± 2.4 nm, 28.1 ± 1.5 nm (Fig. 1b–d), respectively. The differences among the R_a values of the various categories vary statistically ($P < 0.01$). Interestingly, the R_a values of hydrated CS/PCL films ($R_a = 19.1$ nm) differ a little from those of the films measured without hydration ($R_a = 12.5$ nm) while those of highly nano-rough CS/PCL films such as 150 and 100 nm after hydration were markedly lower, at 78.5 and 36.9 nm, respectively. Although the reduced surface roughness of nano-CS/PCL films is shown, the nano-roughness of the surface is maintained (Fig. 1b–d). The

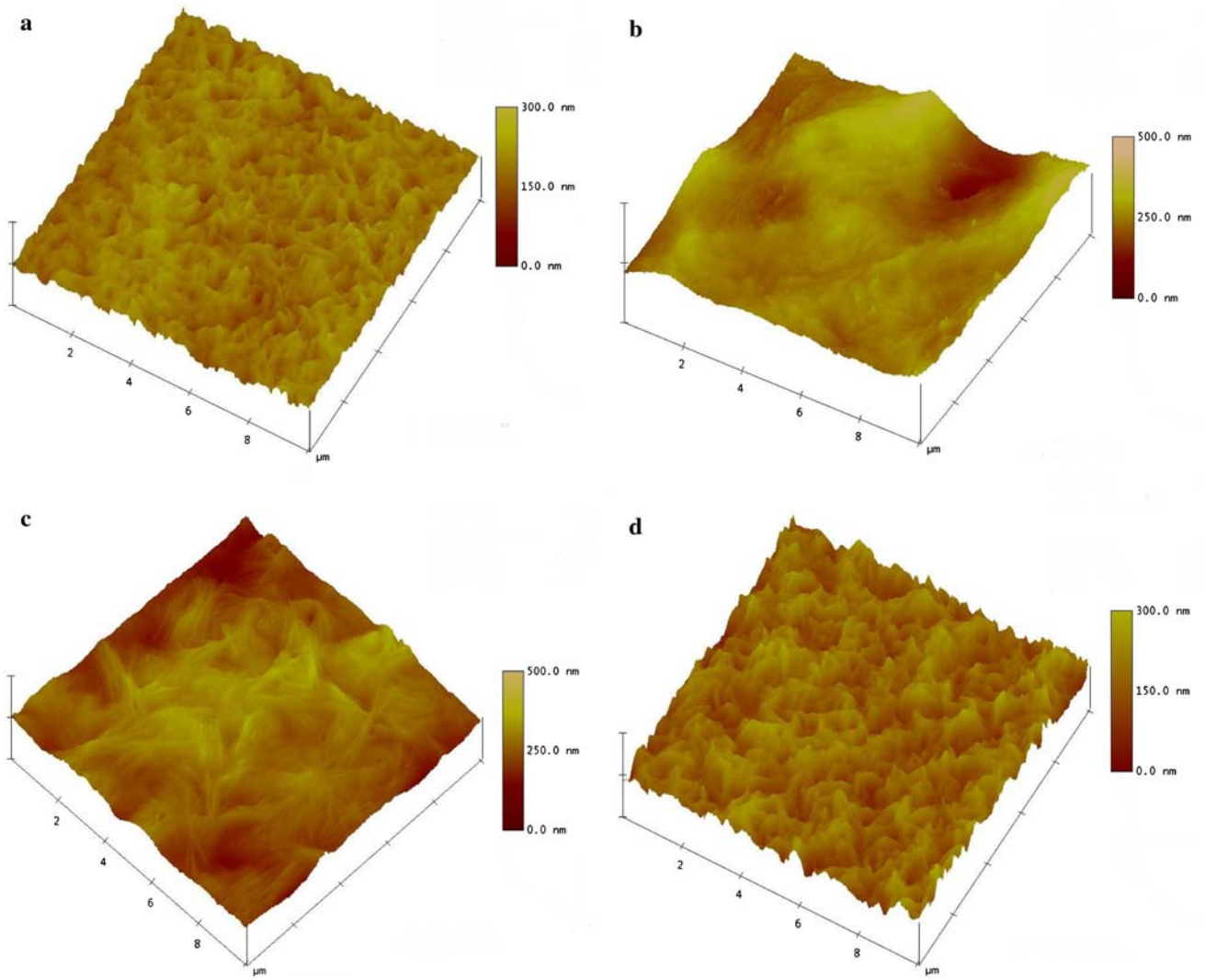

Fig. 1 (**a**–**d**) show the AFM topographies and R_a values of CS/PCL and various nano-roughness of CS/PCL films (**a**–**d**, respectively) after hydration. The vertical axis scales for (**a**, **d**) and (**b**, **c**) are 300 and 500 nm, respectively. (**a**) $R_a = 19.1 \pm 1.5$ nm. (**b**) $R_a = 78.5 \pm 6.3$ nm. (**c**) $R_a = 36.9 \pm 2.4$ nm. (**d**) $R_a = 28.1 \pm 1.5$ nm

large changes in R_a values of the highly nano-rough CS/PCL films upon hydration may be associated with the presence of various non-homogeneous CS surfaces, which may result in the formation of non-uniformly swollen surfaces during hydration, perhaps because the technique employed in this work cannot yield a very uniform nano-structured PCL mold for casting CS surfaces (Diagram 1). Notably, the results of nano-roughness measurements of hydrated films can only provide indirect evidences to depict the nano-CS/PCL surfaces of the films in liquid state.

During the fabrication of the nano-CS/PCL films, the amine groups of CS were firstly crosslinked by genipin to maintain the features of the surface and then reacted with HMDI/THF reagent for PCL grafting. Since the presence of amine groups in CS is essential to support the growth of cells on CS/PCL films, ATR-FTIR transmission spectroscopy was performed to observe the amine groups on the CS surfaces of the aforementioned films (Fig. 2). The transmission peaks at 1640 and 1550 cm^{-1} correspond to amides I and II of CS on the CS/PCL film (curve (a)), respectively, but the transmission intensities of the peak (1640 cm^{-1}) are higher than that of untreated CS (curve (b)).

The viability of HGFs on PCL, CS/PCL and variously rough nano-CS/PCL films after cells had been cultured for 3 and 7 days was determined by both PI fluorescent staining and MTT assay, as described elsewhere [4, 8, 12]. The results of fluorescent staining of the cells were similar to those in the authors' earlier investigations [4, 8, 12]. This study presents PI stains of the nucleus of HGFs on nano-CS/PCL surfaces for 7 days of incubation (Fig. 3). The results reveal that the number of HGFs on a 100 nm

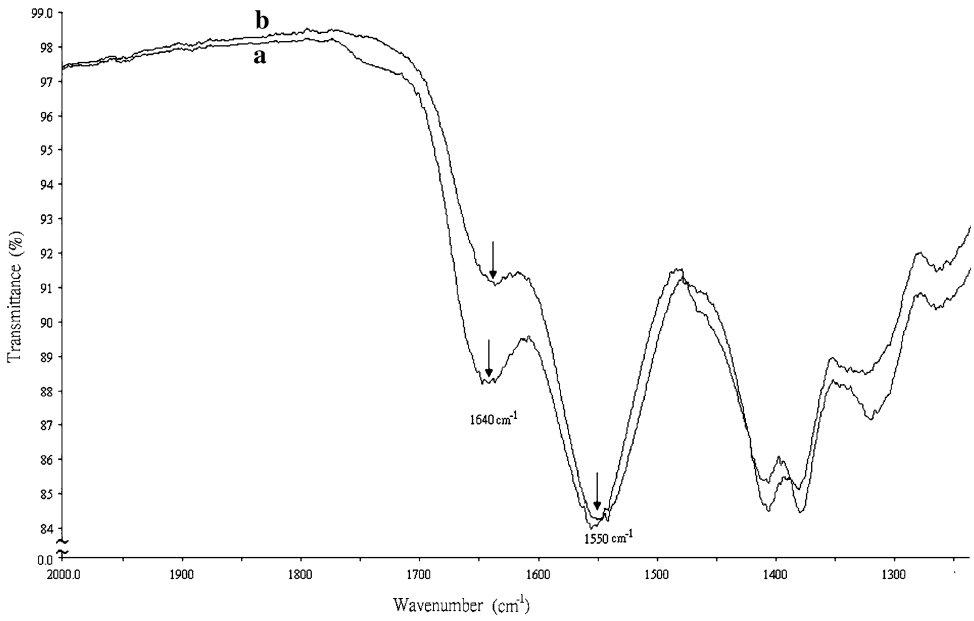

Fig. 2 ATR-FTIR transmission spectra of CS surface of CS/PCL films (**a**) and untreated CS films (**b**). Transmission peaks at 1640 and 1550 cm^{-1} correspond to amide I and amide II, respectively, but the transmission intensity of the peak (1640 cm^{-1}) in curve (**a**) exceeds that of curve (**b**)

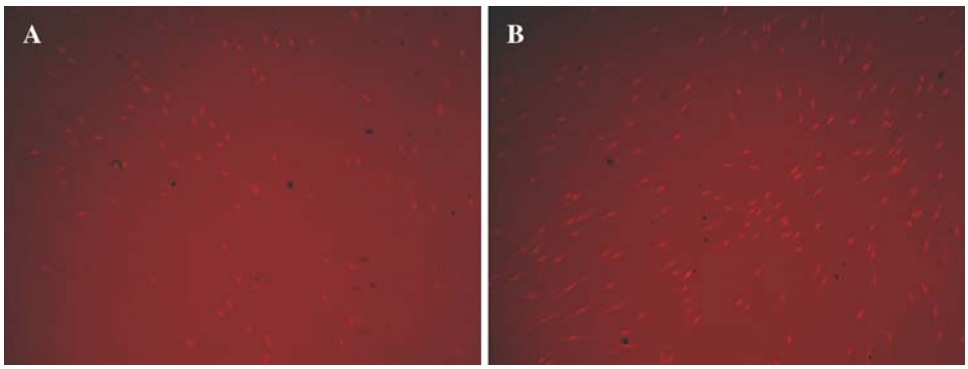

Fig. 3 Fluoresced micrographs of HGFs on (**a**) CS/PCL film (100×) and (**b**) 100 nm nano-CS/PCL films (100×) after 7 days of culturing

nano-CS/PCL film significantly exceeds that on the plain CS/PCL film.

The MTT assay reveals the degree of cell metabolism and the viability of cells [26, 27]. Accordingly, biomaterial researchers have extensively adopted the assay [8, 12, 27]. This work presents MTT assays to analyze the growth and proliferation of HGFs on the films. Importantly, the growths of HGFs on nano-treated and untreated CS/PCL films exceed that of PCL film (Fig. 4), as revealed by the absorbance values of formazan solutions of the films, which demonstrate the effectiveness of grafting CS to PCL on the growth of cells. Interestingly, the absorbance values of MTT assay/and the growth of HGFs on the series of nano-CS/PCL surfaces can be categorized to two groups (2.7, 36.8, 100.0, and 148.0 nm, respectively.) because the MTT values of the group of highly rough nano-CS/PCL films are statistically higher than those of the low-roughness nano-CS/PCL films (Fig. 4) Notably, the numbers of HGFs seeded onto the CS surfaces in the early stage (8 h) were approximately equal about 85% of the total number of incubated cells, and independent of nano-roughness treatment. Hence, the nano-roughness of the surfaces influences the growth rate of HGF on nano-CS films. The rate of proliferation of HGFs on PCL films and the low roughness of CS/PCL films after 7 days of culturing do not differ significantly from those after 3 days (Table 1). In contrast, the growth rates of HGFs on highly nano-rough CS/PCL films after culturing for 7 days with thicknesses of 100 and 150 nm statistically exceed those after 7 days of culturing. The results demonstrate that only highly nano-rough CS/PCL films promote the proliferation of HGFs while low-nano-rough CS/PCL films do not. Notably, unmodified by bio-factors, the growths of human dermal fibroblasts on highly nano-rough nano-PCL films with an R_a value of 106 nm do not promote cell growth and proliferation above those of the untreated films [28], suggesting that the highly nano-rough treatment enhances the growth and proliferation of fibroblasts only on bioactive surfaces (such as CS) herein.

Recently, decreasing proliferation rates of HGFs with increasing surface roughness in micrometer scale of roughness (larger than 1 μm) of substratum is reported [29].

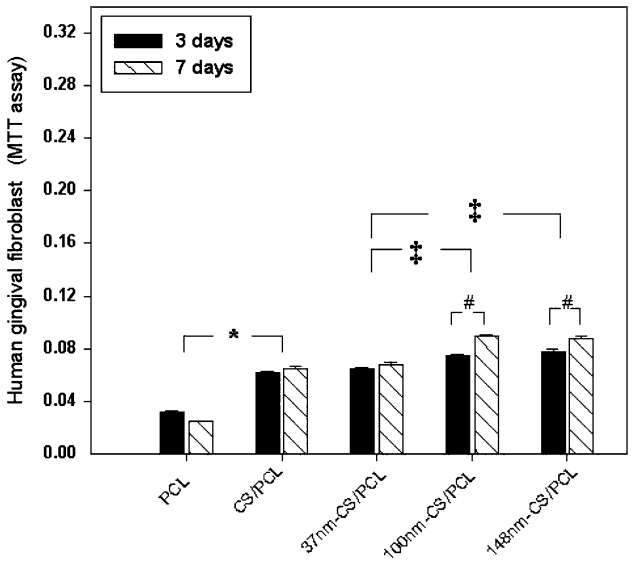

Fig. 4 Viability tests of HGFs grown on PCL, CS/PCL, and nano-CS/PCL films with various R_a values, revealed by the MTT assay following 3 and 7 days of culturing. The absorbance values of the assay of the films are shown and statistically analyzed. The values of the films with CS grafting are significantly higher ($*P < 0.001$) than those of the PCL films for both periods of culturing. Furthermore, the absorbance values of the nano-CS/PCL films with R_a values of 100 and 148 nm are significantly higher ($**P < 0.001$) than those of the films of the CS/PCL and nano-CS-PCL with an R_a of 36.8 nm for both periods of culturing. The values of the nano-CS/PCL films on the 7 day of culturing with R_a values of 100 and 148 nm are significantly higher than ($***P < 0.001$) those on the third day. (All presented data are mean ± SD, $n = 3$)

Table 1 Relative growth rates of HGFs on various surfaces after 3 and 7 days of culturing

Cultural day	Relative growth rates of HGFs (%)	
	3 days	7 days
PCL	17.4 ± 0.6*	13.5 ± 0.7*
CS/PCL	34.3 ± 1.1*	35.9 ± 1.6*
36.8 nm-CS/PCL	35.7 ± 1.4**	37.3 ± 1.5**
100 nm-CS/PCL	41.2 ± 1.1**	49.1 ± 1.5**
148 nm-CS/PCL	42.9 ± 1.6**	48.6 ± 1.9**
Culture plate	100	128.8 ± 4.5

The growth rates on CS/PCL films are significantly higher than those on PCL films (*) on both cultural periods (* $P < 0.001$). Additionally, the growth rates on nano-CS/PCL films with 100 and 148 nm roughness are significantly higher than other CS/PCL films (**) on both cultural periods (** $P < 0.001$)

Note: The rates are the ratio of absorbance values of MTT assay of various surfaces to that of the cell culture plate; the absorbance value for the culture plate is assigned as 100% for 3 days of culturing

However, aforementioned results may not hold in a nanometer scale roughness surfaces. The surface architectures of the vascular walls are on the nanometer scale [9], so mimicking the nano-structure of the surface of the vascular walls to stimulate cell responses is an effective means of reconstructing tissues. More recently, quantified analysis the correlating between surface chemistry and nano-roughness of surfaces and its relation to cell proliferations has been investigated [30, 31]. Those investigations suggest that nano-roughness surfaces enhance protein such as fibronectin adsorption and increase the surface energy on the PU and titanium surfaces, respectively, that increase cyto-compatibility and result in cell adhesion and growth [30, 31].

Methods for constructing the nano-roughness topography of biomaterials and the positive effects of a nano-rough surface on cell growth or function have recently been examined in the laboratories of the authors and others [8, 9, 17, 18]. For example, methods for constructing a nano-structured surface on various biomaterials such as PMMA, PU and PLGA [9, 17, 18, 30, 31], including photolithography [32] and PLGA casting, [17] have been developed. Notably, the aforementioned nano-roughness surfaces were all constructed on hydrophobic synthetic polymers. As is well known, this work may be the first to construct a series of variously different nano-rough CS, a natural bioactive polymer, crosslinked with genipin to maintain the nano-roughness of the surface.

The degree of roughness of nano-surfaces (R_a) on synthetic biopolymers that increases the growth of HUVECs, smooth muscle cells or adhesion of fibroblasts ranged from 30 to 40 nm [8, 17, 18]. According to R_a values of hydrated nano-PCL/CS films of this work, the acceleration of the growths of HGFs on highly nano-rough CS/PCL films are consistent with the results in other studies although they cultured different types of cell, such as HUVEC or smooth muscle cells [8, 17, 18, 33, 34]. Although the factors of aforementioned protein adsorption and surface energy may different in different nano-roughness, the detailed mechanisms that are responsible for the significant differences in cell growths and proliferations between the high and low nano-rough CS/PCL films (Fig. 4) must be further examined.

4 Conclusion

A series of variously nano-rough CS/PCL surfaces are fabricated by grafting variously nano-rough CS surfaces onto PCL polymers. CS grafting promotes the growth of HGFs on the PCL films for two periods of culturing. Notably, highly nano-rough CS surfaces further promote the growth and proliferation of HGFs in vitro on nano-CS/PCL films above those on low-nano-rough surfaces. Grafting bioactive factors with a high nano-rough treatment on biodegradable polymer scaffolds or surfaces may be employed to engineer soft tissues.

Acknowledgments The authors would like to thank the National Science Council of the Republic of China, Taiwan, for financially supporting this research under Contract Nos. NSC-93-2314-B-002-233 and NSC-93-2213-E-224-009. Ted Knoy is appreciated for his editorial assistance.

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